IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:
Boyse et al.

U.S. Patent No.: 5,004,681

For: ISOLATION AND PRESERVATION OF FETAL AND NEONATAL HEMATOPOIETIC STEM AND PROGENITOR CELLS OF THE BLOOD

Group Art Unit: 1808

Examiner: Susan M. Dadio

Reexam. No. 90/003182

Reexam. Request Filed: August 30, 1993

Attorney Docket No.: 6287-021

SECOND DECLARATION OF DR. IRWIN D. BERNSTEIN

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

S I R:

I, DR. IRWIN D. BERNSTEIN, do declare and state that:

1. I am a citizen of the United States residing at 4949 Stanford Avenue Northeast, Seattle, Washington 98105.

2. I received the degree of Medical Doctor from New York University, New York, New York in 1967. I received the degree of Bachelor of Sciences in Biology from Trinity College, Hartford, Connecticut in 1963.

3. I presently hold the positions of Professor of Pediatrics, and Director of Division of Pediatric Hematology of the University of Washington School of Medicine,

EXPRESS MAIL CERTIFICATION

"Express Mail" label No. TB 294 001 576 US Date of Deposit July 20, 1994
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

ANDREA SILVERMAN
(Type or print name of person mailing paper or fee)
(Signature of person mailing paper or fee)

Seattle, Washington; and Member and Program Head of Pediatric Oncology, Fred Hutchinson Cancer Research Center, Seattle, Washington.

4. My complete academic background, publications, professional experience and honors are set forth in my *curriculum vitae*, a copy of which is annexed as Exhibit A to the Declaration of Irwin D. Bernstein which was filed on February 4, 1994 for the above-identified reexamination (hereinafter the "first Bernstein Declaration").

5. My present employment involves both research and clinical practice. My research focuses on hematopoietic stem cells (both normal and malignant), and antibody-targeted therapy for hematopoietic malignancies. I am presently conducting and have in the past conducted research directed to the isolation and characterization of bone marrow cells required for hematopoietic reconstitution in humans. I routinely interact in my research activities with the bone marrow transplant group at the Fred Hutchinson Cancer Research Center. I have been conducting research directly relating to hematopoietic cells since approximately 1979. My clinical practice is mainly general pediatric oncology, but I also have been responsible for some patients undergoing bone marrow transplantation and some patients undergoing transfusion with autologous peripheral blood mononuclear cells. I am the author or co-author of over 190 research, technical or proprietary publications of which more than 50 pertain to hematopoietic reconstitution, hematopoiesis or hematopoietic stem or progenitor cells. In particular, I have authored or co-authored approximately eighteen publications directly pertaining to hematopoietic reconstitution.

6. I have read and am familiar with United States Patent No. 5,004,681 ("the '681 Patent"). I have read and am familiar with the Request for Reexamination of the '681 Patent filed by Cryo-Cell International, Inc., the Order

Granting Request for Reexamination dated November 5, 1993, Patent Owner's Statement Under 37 C.F.R. § 1.530 filed February 4, 1994, Requester's Reply to Patent Owner's Statement dated March 17, 1994, and the Office Action in Reexamination dated May 20, 1994. I have read and am familiar with the following 12 references, relied upon by the Examiner in the Office Action dated May 20, 1994:

Shope et al., Proceedings of the Society for Experimental Biology and Medicine, 157:326-329 (1978) ("Shope et al.")

Moretti et al., Fetal Liver Transplantation, pp. 121-133, copyright 1985 by Alan R. Liss, Inc., Prog. Clin. Biol. Res. 193:121-133 ("Moretti et al.")

Fliedner and Calvo, Fetal Liver Transplantation, Current Concepts and Future Directions, "Proceedings of the First International Symposium on Fetal Liver Transplantation, Pesaro, Italy, September, 1979," pp. 305-309 ("Fliedner et al.")

Ende, Virginia Medical Monthly, 99:276, March, 1972 ("Ende")

Löwenberg, Bob, Fetal Liver Cell Transplantation, Role and nature of the fetal haemopoietic stem cell, Ultgeverij Waltman-Delft, 1975, Section 1.3.6, "Fetal liver cell transplantation in man," pp. 25-28 and p. 36 ("Löwenberg")

Ueno et al., Exp. Hematol. 9:716-722 (August 1981) ("Ueno et al.")

Prindull et al., Acta Paediatr. Scand., 67:413 (1978) ("Prindull et al.")

Korbling et al., Blood 67(2):529-532 (1986) ("Korbling et al.")

Valeri, in *Blood Banking and the Use of Frozen Blood Products*, Ch. 1, CRC Press, pp. 1-7 (1976) ("Valeri")

Karp et al., Am. J. Hematol. 18:243-249 (1985) ("Karp et al.")

Gorin, Clin. Haematol. 15:19-48 (1986) ("Gorin")

Nakahata and Ogawa, J. Clin. Invest. 70:1324-1328 (1982) ("Nakahata et al.")

7. I have read claims 1-9 of the '681 Patent. The invention described and claimed in the '681 Patent is a composition of viable human neonatal or fetal

hematopoietic stem cells derived from the blood in combination with cryopreservative. In some claims, the composition also contains, in addition to such cryopreserved stem cells, viable human neonatal or fetal hematopoietic progenitor cells, whole neonatal or fetal blood, or anticoagulant. In other claims, the cryopreservative is specified, or the stem or progenitor cell is characterized by certain abilities.

8. I make this Declaration to supplement the remarks I made in the first Bernstein Declaration, to explain further the basis for my conclusion that the claimed invention of the '681 Patent was not obvious to one of ordinary skill in the art at about the filing date of the application leading to the '681 Patent (November 12, 1987).

9. Hematopoietic stem and progenitor cells are the cells from which the mature functional cells circulating in the blood derive. Stem cells are the most primitive cells in the hematopoietic lineage; they have extensive proliferative capacity and the ability to generate other stem cells as well as to differentiate into the progenitor cells, which in turn can differentiate into the mature cells. The mature cells include erythrocytes (red blood cells), granulocytes, monocytes/macrophages, megakaryocytes, T cells, B cells, and non-T, non-B cells. In progressing through the hierarchy of hematopoietic cells from stem cells to progenitor cells to mature cells, the cells have progressively more restricted differentiation capacity, *i.e.*, less ability to produce mature cells of different types within the different blood cell lineages. The blood cell lineages consist of both the myeloid (including the mature cells that are erythroid cells (*e.g.*, red blood cells), granulocytes, monocytes/macrophages, and megakaryocytes) and lymphoid (including the mature cells that are T-, B-, and non-T, non-B cells) lineages. Stem cells are pluripotent in that they have the greatest potential, by differentiation, to produce the various cells of the different blood cell lineages. Progenitor cells have more limited

multipotentiality and a lesser degree of proliferative capacity. The direct precursors to mature cells are unipotent, in that they can only produce their own kind. The most immature human hematopoietic stem cell is the cell with long-term, marrow repopulating ability that is able to effect hematopoietic reconstitution. I use the term "hematopoietic reconstitution," consistent with its usage in the '681 Patent, to mean "long-term," complete (multilineage) hematopoietic repopulation *in vivo*. It is the stem cell with long-term marrow repopulating ability that, in sufficient amounts, has utility for hematopoietic reconstitution. Cells other than the long-term marrow repopulating stem cell also have been termed stem cells, such as those detected by the CFU-s (spleen colony forming) assay[1] or the ability to give rise to *in vitro* blast cell colonies that can be replated *in vitro* to form secondary colonies containing the different mature blood cell types (such as disclosed by Nakahata et al.). Despite this use of the "stem cell" terminology, it was recognized in the art at about the time the application leading to the '681 Patent was filed, and is presently recognized, that the cells detected by the CFU-s assay and the cells giving rise to *in vitro* blast cell colonies had an unknown relationship to the stem cells with the ability to effect hematopoietic reconstitution. Assays which detect cells that form colonies *in vitro* of mature blood cells are detecting progenitor cells. As set forth in the Definitions section of the '681 Patent,[2] progenitor cells detected by the ability to form

---

1 The CFU-s assay detects murine, not human, cells; it is a colony forming assay done *in vivo* in mice.

2 See col. 8, line 46 to col. 9, line 6.

colonies *in vitro* of mature blood cells include the BFU-E,[3] CFU-GEMM,[4] and CFU-GM.[5] The more different types of mature cells that are produced in a single colony, the broader the potentiality (breadth of differentiation ability) of the colony-forming cells thus detected. Thus, for example, CFU-GEMM are multipotential progenitor cells that are more primitive (earlier in the differentiation hierarchy) than CFU-GM. Since the definitions of hematopoietic stem and progenitor cells are operational, their presence can be determined only by the appropriate functional assay. In particular, the presence of a long-term marrow repopulating stem cell in any particular composition is only reasonably expected if that composition has been shown capable of providing long-term, complete (multilineage) repopulation of the blood components *in vivo* (*i.e.*, hematopoietic reconstitution).

10. Prindull et al. discloses cells which give rise to colonies *in vitro* composed of one type of mature cell, a myelocyte/metamyelocyte. Ueno et al. discloses (i) cells which give rise to colonies *in vitro* composed of one type of mature cell, a granulocyte, (ii) cells which give rise to colonies *in vitro* composed of monocytes/macrophages (a monocyte is the direct precursor to the macrophage, a mature cell type), and (iii) only a few cells giving rise to colonies of both granulocytes and monocytes/macrophages (*i.e.*, CFU-GM) (p. 719, Table 1 and col. 1). It is clear in view

---

3 "BFU-E = burst-forming unit-erythroid. An hematopoietic progenitor cell which is capable of producing a colony of erythroid progeny cells in semi-solid medium" (the '681 Patent at col. 8, lines 49-52).

4 "CFU-GEMM = colony-forming unit-granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte. A multipotential hematopoietic progenitor cell which is capable of producing a colony composed of granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte progeny, in semi-solid medium" (the '681 Patent at col. 8, line 65 to col. 9, line 2).

5 "CFU-GM = colony-forming unit-granulocyte, macrophage. An hematopoietic progenitor cell which is capable of producing a colony composed of granulocyte and macrophage progeny in semi-solid medium" ('681 Patent at col. 9, lines 3-6).

of Paragraph 9 above, that none of these colony-forming cells have the pluripotentiality characteristic of stem cells.

11. Even though the authors of these 1978 (Prindull et al.) and 1981 (Ueno et al.) publications at times term the cells detected by their assays "stem cells," by 1987 it was commonly recognized in the art that these colony-forming cells were progenitor cells since they exhibited the potential to produce *in vitro* colonies composed of mature cells of only one or two types (and thus were not multipotential much less pluripotential). Nevertheless, regardless of whether these colony-forming cells are termed "stem cells" or "progenitor cells," it was commonly known in the art that these colony forming cells were not the stem cells that are capable of carrying out hematopoietic reconstitution. Indeed, even as early as 1976, Richman et al., 1976, Blood 47:1031-1039, attached hereto as Exhibit H and discussed in more detail in Paragraph 27 below, discloses what the authors term "committed granulocytic stem cells[6] which form colonies in semisolid medium (CFU-C)" (p. 1031), derived from human peripheral blood, that are not deemed by the authors to be pluripotential stem cells, since they state that "[a]s yet, no direct assay for the pluripotential stem cell is available in man [citations]" (p. 1031).

12. Indeed, scientific evidence demonstrating that colony-forming cells such as disclosed by Prindull et al. and Ueno et al. could be physically separated from the cells that were capable of effecting hematopoietic reconstitution, was commonly known and available in the art prior to the filing date of the application leading to the '681 Patent. This evidence is described in Paragraphs 13-19 hereinbelow.

---

[6] By about the time of the filing of the application leading to the '681 Patent, these cells were commonly regarded as progenitor cells.

13. Attached hereto as Exhibit A is the publication by Rowley et al., 1985, Exp. Hematol. 13:295-298 ("the Rowley Publication"). The Rowley Publication evidences the fact that, by 1985, it was known in the art that progenitor cells, even the multipotential (and thus relatively immature) CFU-GEMM, were not representative of the stem cell that was capable of effecting hematopoietic reconstitution. The authors incubate bone marrow cells with 4-hydro-peroxycyclophosphamide (4-HC), which eliminated most detectable progenitor cells but was used at concentration which was known not to destroy the ability to effect hematopoietic reconstitution.[7] The authors of the Rowley Publication cite a previous publication describing a successful hematopoietic reconstitution using bone marrow in which virtually all detectable committed progenitor cells (BFU-E and CFU-GM) were absent, thus showing that the presence of committed progenitor cells does not correlate with the presence of the long-term marrow repopulating stem cells (that are able to effect hematopoietic reconstitution), for which stem cells the authors acknowledge there is no *in vitro* assay:

> The postincubation survival of committed progenitor cells was not predictive of the marrow repopulating ability of the treated marrow, apparently because committed progenitor cell survival following in vitro incubation with 4-HC does not correlate with the survival of the currently undetectable hematopoietic stem cell. Therefore, successful marrow reconstitution could not be predicted prior to reinfusion of the treated marrow based upon the committed progenitor cell content of the marrow samples.

(p. 295, col. 2; emphasis added).

Since it was thus known in the art that committed progenitor cell content did not reflect the ability to effect hematopoietic reconstitution, the authors of the Rowley Publication investigated whether the more primitive, multipotential CFU-GEMM progenitor cell,

---

[7] 4-HC was used in an attempt to eliminate malignant cells from the marrow *in vitro*, prior to infusion into the patient as part of a treatment regimen.

which shares certain characteristics with stem cells, might actually be the stem cell with the ability to effect hematopoietic reconstitution, or at least indicative of the presence of such stem cell:

> The multilineage progenitor cell (CFU-GEMM) demonstrates some characteristics possessed by the hematopoietic stem cell including the potential to develop along multiple cell lineages [4], a capacity for self-renewal [5], and a proposed normally quiescent proliferative stage [6]. . . . It is conceivable therefore that if this cell is directly responsible for marrow reconstitution or representative of the hematopoietic stem cell that is, it would survive incubation with 4-HC in the dose ranges used in the clinical trial described above.

((p. 295-296, col. 1).

The results showed that both the detectable committed progenitors and the CFU-GEMM displayed a dose-dependent sensitivity to 4-HC treatment (Table 1, p. 296), in contrast to the reported insensitivity of the long-term marrow-repopulating stem cell to 4-HC treatment. The results indicated that the CFU-GEMM is not the stem cell which can effect hematopoietic reconstitution, and that its presence does not reliably predict the presence of such a long-term marrow repopulating stem cell. As the authors state:

> . . . the demonstrated toxicity of 4-HC for the CFU-GEMM suggests that this cell is not the hematopoietic stem cell responsible for marrow repopulation, since very few CFU-GEMM are detectable following exposure to 4-HC at concentrations still allowing for successful marrow reconstitution.

(p. 297, col. 1).

They further state:

> The present results do question the value of the CFU-GEMM assay as a predictive assay for marrow reconstituting ability following in vitro 4-HC treatment. The dose-dependent sensitivity and the virtual absence of detectable CFU-GEMM at dose levels allowing successful

> marrow reconstitution precludes the use of this assay as a probe for the quantity of viable stem cells a marrow sample may contain, at least after some in vitro treatments.

(p. 297, col. 2).

The Rowley Publication thus shows that even the multipotential cells capable of forming multilineage colonies *in vitro* are physically distinct and can be separated from the stem cells which have the ability to effect hematopoietic reconstitution.

14. Attached hereto as Exhibit B is the publication by Gordon et al., 1985, Leukemia Research 9:1017-1021 ("the Gordon Publication"). The Gordon Publication presents additional evidence showing that cells with the ability to form *in vitro* colonies of granulocytes and macrophages (GM-CFC)[8] are distinct from the stem cells with the ability to effect hematopoietic reconstitution. Similar to the Rowley Publication, the authors treat human bone marrow cells with 4-HC (used at lower concentrations than in the Rowley Publication) and assess survival of GM-CFC and type I blast colony-forming cells.[9] The results showed that GM-CFC were much more sensitive to 4-HC than the type I blast colony-forming cells. Their results and those of others lead the authors to conclude that:

> It is now well established that the drugs [4-HC and ASTA-Z-7557] are toxic to human GM-CFC, BFU-E and GEMM-CFC as well as to leukaemic colony-forming cells [citations]. Thus, comparison of the *in vitro* results with the performance of marrow reinfused after treatment with 4-HC shows clearly that the current assays for hematopoietic progenitor cells do not adequately predict the capacity of the marrow to engraft.

(p. 1018, cols. 1-2, emphasis added).

---

[8] Also known in the art as CFU-GM.

[9] Type I blast colony forming cells are cells detected in a type of *in vitro* colony forming assay; see the Gordon Publication at p. 1017, col. 2 to p. 1018, col. 1.

Even with respect to the more 4-HC resistant type I blast colony-forming cells, which the results in the Gordon Publication suggest are progenitor cells more primitive than CFU-GM, the authors indicate that these cells cannot be reasonably relied upon as representative of the survival of long-term marrow repopulating stem cells:

> However, it will be necessary to extend these preliminary studies to higher, clinically used doses of 4-HC before the results can be used to suggest that the blast colony-forming cells may provide a better indication than the other colony assays of the transplantability of human marrow after it has been manipulated *in vitro*.

(p. 1018, col. 2).

15. Attached hereto as Exhibit C is the publication by Siena et al., 1985, Blood 65(3):655-662 ("the Siena Publication"). The Siena Publication presents additional results demonstrating that cells detected by *in vitro* colony forming assays are distinct from the stem cell that affords hematopoietic reconstitution. The Siena Publication discloses that treatment of human bone marrow with 4-HC at suitable concentrations eliminates the majority of CFU-Mix[10], as well as the less primitive BFU-E and CFU-GM cells, while sparing more primitive cells capable of subsequently giving rise to progenitor cells in long-term marrow culture. The authors note CFU-Mix to be "a progenitor cell with self-renewal characteristics [citations] that has been considered the putative primitive stem cell" (p. 660, col. 1), and state that their results, showing that bone marrow depleted of CFU-Mix can carry out hematopoietic reconstitution, "indicates that CFU-Mix does not represent the stem cell responsible for hematopoietic reconstitution of the transplanted host" (p. 660, col. 1).

---

[10] CFU-Mix are multipotential progenitor cells capable of generating *in vitro* colonies containing erythroid, granulocyte, monocyte, and megakaryocytic cells (the Siena Publication, p. 656, col. 1). CFU-Mix are also known in the art as CFU-GEMM.

16. Attached hereto as Exhibit D is the publication by Yeager et al., 1986, N. Engl. J. Med. 315(3):141-147 ("the Yeager Publication"). The Yeager Publication reports clinical results in humans demonstrating that treatment of bone marrow with 60-100 μg/ml of 4-HC does not destroy the ability of the treated bone marrow to effect successful hematopoietic reconstitution, although CFU-GM are virtually eliminated in the treated bone marrow (see Table 2 on page 143, and page 145, second full paragraph). The Yeager Publication thus demonstrates that CFU-GM are distinct from and can be separated from the stem cell that effects hematopoietic reconstitution.

17. Attached hereto as Exhibit E is the publication by Kaizer et al., 1985, Blood 65(6):1504-1510 ("the Kaizer Publication"). The Kaizer Publication reports clinical results in humans demonstrating that treatment of bone marrow with 4-HC concentrations that completely eliminated granulocyte and macrophage colony-forming cells[11] does not destroy the ability of the treated bone marrow to afford hematopoietic reconstitution (see Fig. 1 on p. 1507 and related text; Table 1 on p. 1505 and Fig. 2 on p. 1508). The authors acknowledge that there is no *in vitro* assay for the long-term marrow repopulating stem cell, and indicate that measurements of CFU-C are not satisfactory indications of the ability to effect hematopoietic reconstitution:

> The pluripotent lymphohematopoietic stem cell cannot currently be measured in human bone marrow. Most clinical investigations of autologous BMT have, therefore, used measurements of CFU-Cs to assess the quality of the marrow cells infused. This use of CFU-Cs suffers from a number of problems. First, the CFU-C is several stages further along the differentiation pathway than the pluripotent stem cell [citation]. Second, most clinical studies of autologous BMT [bone marrow transplants] have shown that the correlation between the number of CFU-Cs infused and the rate of recovery is minimal at best [citations]. Finally, this study

---

[11] The Kaizer Publication terms these cells "CFU-C"; they are actually CFU-GM.

> has shown that satisfactory hematologic recovery can be obtained, despite inhibition of all measurable CFU-Cs.

(paragraph spanning pp. 1508-1509).

18. Attached hereto as Exhibit F is the publication by Andrews et al., 1986, Blood 68(5):1030-1035 ("the Andrews Publication"), of which I am a coauthor. The Andrews Publication describes experiments demonstrating that hematopoietic *in vitro* colony-forming cells are distinguishable from more primitive hematopoietic cells that are precursors to the colony-forming cells, based on differences in proteins expressed on the cell surface. Human bone marrow was treated with monoclonal antibody L4F3 and complement. This antibody (which recognizes what is now termed the CD33 antigen), in the presence of complement, eliminated cells expressing the L4F3 antigen; virtually all immature colony-forming cells, including CFU-GM, BFU-E, and CFU-Mix, were eliminated (p. 1031, col. 1, first full paragraph). CFU-Meg[12] were also eliminated. After elimination of the *in vitro* colony forming cells, the remaining cells were able to generate CFU-GM, BFU-E and CFU-Mix when cultured in long-term marrow cultures (Table 1, p. 1032). Fluorescence activated cell sorting (FACS) demonstrated that cells expressing the L4F3 antigen (expressing CD33; L4F3-positive cells) included almost all CFU-Mix, CFU-Meg, and BFU-E, as well as the majority of CFU-GM (thus, including both multipotential and unipotential progenitor cells) (p. 1031, third full paragraph). The L4F3-negative cells (that did not express L4F3 antigen), which were separated by FACS from the above enumerated L4F3-positive *in vitro* colony forming cells, contained precursors to CFU-GM as demonstrated by the ability of the L4F3-negative cells, similar to that of unseparated marrow cells, to generate CFU-GM in long-term culture (p. 1033, Table 3). The results thus show that virtually all *in vitro* colony-forming cells in bone

---

[12] A cell detected by its ability to produce a colony of megakaryocytes *in vitro*.

marrow express the L4F3 antigen, while more immature cells which are precursors to the colony-forming cells do not. Clearly, the *in vitro* colony forming cells cannot represent the most immature hematopoietic cell, the long-term marrow repopulating stem cell.[13]

19. Attached hereto as Exhibit G is the publication by Jacobsen et al., 1979, Cell Tissue Kinet. 12:213-226 ("the Jacobsen Publication"). The Jacobsen Publication demonstrates that as early as 1979 it was reported that human bone marrow cells which give rise *in vitro* to colonies composed of granulocyte and macrophage cells (termed "CFU-c")[14] could be physically separated from cells that appeared to be precursors to such CFU-c (see p. 225, third to fifth full paragraphs). Thus, it is clear that even as early as 1979, the art did not believe CFU-GM to be the earliest (most primitive) hematopoietic cells in the hematopoietic cell hierarchy.

20. The presence of the cord blood cells detected by Nakahata et al. also does not reasonably predict the presence of the stem cell with the ability to effect hematopoietic reconstitution. Nakahata et al. discloses cord blood cells that are detected by their ability to produce, *in vitro*, after replating, multipotential colonies of hematopoietic cells including progenitors to colonies of granulocyte-erythrocyte-macrophage-~~macrophage~~-megakaryocyte (GEMM) cells (Table II, p. 1327). However, no lymphoid colonies were identified (either before or after replating). Even Nakahata et al. note that "[w]e did not identify lymphoid colonies in the replating experiments of blast cell colonies." (p. 1327, col. 2, first full paragraph.) The authors also state that "[t]his human blast cell colony assay may provide a method for quantitation of more primitive

---

13 This has subsequently been confirmed, since marrow depleted of CD33-bearing cells (thus depleting the marrow of virtually all colony-forming cells) has been shown able to afford hematopoietic reconstitution (Robertson et al., 1992, Blood 79:2229-2236).

14 These CFU-c are CFU-GM.

hematopoietic stem cells than progenitors for GEMM colonies (CFU-GEMM) in man," (p. 1324), and "[f]urther improvement of the replating conditions is necessary for confirmation of the self-renewal capacity of human blast cell colonies" (p. 1328). As warranted by their data, nowhere in this publication do the authors suggest that their assay detects the stem cell with the ability to effect hematopoietic reconstitution. Nakahata et al. does not disclose cells which lead me, or would lead one of ordinary skill in the art, to reasonably expect that human neonatal or fetal blood contained the long-term marrow-repopulating stem cells so as to have utility for hematopoietic reconstitution.

21. In view of Paragraphs 9-20 above, I conclude, and one of ordinary skill in the art (at about the time of the filing of the application leading to the '681 Patent) would conclude, that the presence of cells in cord blood which form colonies *in vitro*, before or after replating, does not reasonably predict the presence of stem cells in cord blood that are capable of carrying out human hematopoietic reconstitution.

22. I have discussed Shope et al. in Paragraphs 10-12 of the first Bernstein Declaration. As explained therein, this reference does not even suggest the presence of cord blood stem cells, does not motivate their formulation with cryopreservative, and thus does not render obvious the claims of the '681 Patent. Paragraphs 10-12 of the first Bernstein Declaration also demonstrate why it cannot be concluded that the procedures disclosed by Shope et al. inevitably and necessarily yield a fraction of cord blood containing viable stem cells that is combined with cryopreservative.

23. As stated above, I have reviewed the combinations of references upon which the Examiner relies in the Office Action dated May 20, 1994, in rejecting the claims of the '681 Patent as obvious. For the reasons stated in the first Bernstein Declaration and further as stated below, I conclude that none of these combinations of

references suggest or motivate the cryopreservation of any composition containing human neonatal or fetal blood stem cells.

24. As I discuss in Paragraphs 9-19 above, the human cord blood cells reported by Ueno et al. and Prindull et al. are progenitor cells, based on their ability to produce colonies *in vitro* composed of mature blood cells of limited type and one skilled in the art would clearly recognize them as such and as distinct from blood stem cells and very different from the stem cell capable of effecting hematopoietic reconstitution. For the reasons discussed in Paragraph 20 above, cells detected by the *in vitro* colony assay of Nakahata et al. do not lead me or one of ordinary skill in the art to reasonably expect that human neonatal or fetal blood contained long-term marrow repopulating stem cells so as to have utility for hematopoietic reconstitution.

25. The presence of stem cells with long-term marrow repopulating ability in bone marrow or fetal liver (as disclosed by Moretti et al., Fliedner et al., Lõwenberg and/or Gorin), or adult peripheral blood regenerating after chemotherapy (as disclosed by Korbling et al. or Karp et al.) sheds no light on whether amounts of cord blood stem cells with long-term marrow repopulating ability exist in cord blood so as to have use for hematopoietic reconstitution. The state of the art at about the time of the filing of the application leading to the '681 Patent was such that, without prior knowledge of the presence of stem cells that are able to effect hematopoietic reconstitution, *in vitro* assays do not allow a reasonable prediction of the presence of such stem cells or of utility for hematopoietic reconstitution. Furthermore, it was known in the art at about the time of the filing of the application leading to the '681 Patent that even the different stem cell sources known to contain long-term marrow repopulating stem cells (*e.g.*, bone marrow, "rebounding" adult peripheral blood, and fetal liver) were not interchangeable with

respect to their utility for hematopoietic reconstitution. For example, the lack of accessibility and availability of fetal liver entailed by the necessity to acquire this source from aborted fetuses, was problematic with respect to both the initial availability of fetal liver and the acquisition of a large enough fetal liver to yield a sufficient number of stem cells to achieve hematopoietic reconstitution. The foregoing, and numerous clinical failures or problems (*e.g.*, graft-versus-host-disease (GVHD)) associated with the use of fetal liver for hematopoietic reconstitution had led by 1987 to diminished interest by the medical community in use for hematopoietic reconstitution. Indeed, bone marrow transplantation was, as of the filing date of the application leading to the '681 Patent, the most favored approach for general use in achieving hematopoietic reconstitution, though not without its own problems (see Paragraph 35 hereinbelow). Success with adult peripheral blood mononuclear cells was generally viewed as achievable only under limited circumstances -- for autologous use, collected when the very low levels of stem cells present in normal adult peripheral blood were believed increased in cancer patients due to a "rebound effect" involving the increase of certain blood components following chemotherapy (see Paragraphs 26-28 hereinbelow).

26. In particular, in this and the following Paragraphs 27-30, I address the Examiner's comments in the Office Action dated May 20, 1994, to explain the evidence showing that the peripheral blood cell composition used for hematopoietic reconstitution by Korbling et al. was altered by the patient's disorder and/or chemotherapy, and why it would not be obvious to substitute cord blood for the adult peripheral blood taught by Korbling et al. The use of adult peripheral blood mononuclear cells is exemplified by Korbling et al., relied upon by the Examiner. Korbling et al. describes the hematopoietic reconstitution of a 38 year-old patient with Burkitt's lymphoma through the use of

autologous adult peripheral blood mononuclear cells collected from the patient and cryopreserved. It is to be noted that the blood cells used were adult peripheral blood mononuclear cells obtained from a lymphoma patient after chemotherapeutic treatment of the patient. Evidence to support the conclusion that the cells disclosed by Korbling et al. are altered by the patient's disorder and/or chemotherapy is found within the reference itself. The authors explicitly acknowledge that high numbers of hematopoietic precursor cells were obtained and infused due to the fact that the blood was obtained from the patient just two weeks after completion of chemotherapy, probably during an expansion of blood stem and progenitor cells in response to blood cell destruction caused by chemotherapy (page 532, column 1):

> "The high yield of leukapheresis-derived hemopoietic precursor cells reflects an expansion of the blood stem cell pool at the time of stem cell harvest. Since leukapheresis started just 2 weeks after completion of chemotherapy, stem cell harvest was probably performed during a chemotherapy-induced blood stem cell overshoot as described first by Richman et al.[17] [Richman et al., 1976, Blood 47:1031]."

Whether the reported usefulness of adult peripheral blood stem cells from patients with lymphoma or leukemia was due to their chemotherapy or to their cancer was unclear; however, it was clear that the properties of the peripheral blood in these patients, that gave rise to this usefulness, were not applicable to normal individuals.

27. Attention is also directed to the publication by Richman et al., 1976, Blood 47:1031 ("the Richman Publication"), attached hereto as Exhibit H, which is cited by Korbling et al. in the passage quoted in the Paragraph hereinabove. The Richman Publication measures the amounts in adult peripheral blood of granulocytic colony-forming units, which cells they termed "CFU-C"[15]. The Richman Publication shows

---

[15] These CFU-C are progenitor cells; see Paragraph 9 hereinabove.

that serial changes in the amounts of adult human peripheral blood CFU-C occur in patients with solid tumors subjected to intermittent chemotherapy (p. 1031, last paragraph). Prior to chemotherapy, there was no difference in CFU-C levels in normal donors relative to the patients. After chemotherapy, the median CFU-C concentration increased to a significantly higher value [23 (colonies per 2 x 10^5 mononuclear cells) as opposed to 9.5 for normal donors or 6 for patients prior to chemotherapy) (Table 2 on p. 1034, p. 1035, first paragraph); in contrast, the number of circulating mononuclear cells remained unchanged by chemotherapy. Thus, after chemotherapy, the progenitor cell content in adult peripheral blood is much higher than that present in normal adult peripheral blood. In view of this "rebound effect," the authors state that "although no direct assay is available for quantitating pluripotential stem cells in man, one can speculate that a parallel increase in the primitive and in the committed stem cells might occur after cyclophosphamide/adriamycin therapy." On page 1037, last paragraph, the authors discuss their results, indicating, by analogy to bone marrow CFU-C content, the unfeasibility of obtaining sufficient cells from normal adult peripheral blood or in patients prior to chemotherapy:

> Prior to chemotherapy, the number of CFU-C obtained from the peripheral blood by pheresis was 3.2 per 2 x 10^5 mononuclear cells. Since a 1-liter pheresis took 40 min and yielded 1.4 x 10^9 mononuclear cells, we estimated that a bone marrow equivalent dose of CFU-C would necessitate clearance of 442 liters of blood requiring 296 hr.

(p. 1037, last paragraph).

Clearly, collecting such a large amount of blood from a patient over such a long period of time cannot practically be done.

28. Karp et al., also relied upon by the Examiner, also reports the use of autologous adult peripheral blood mononuclear cells collected from leukemia patients after

chemotherapy[16] in attempted hematopoietic reconstitution. The authors note that in view of the fact that bone marrow transplantations have curative potential for the leukemia patients, "the ultimate usefulness of this therapy remains unclear," but that it appears to offer an important option for those patients without a compatible donor or who are unable to sustain an allograft (p. 248, first paragraph).

29. In view of the fact that success in using adult peripheral blood mononuclear cells for hematopoietic reconstitution was commonly recognized in the art by about the time of the filing of the application leading to the '681 Patent as achievable generally only when using adult peripheral blood collected during the "rebound effect" after chemotherapy, it was commonly believed in the art that the stem cell with the ability to effect hematopoietic reconstitution circulated at unsuitably low levels in normal adults, but that levels of this stem cell increased in peripheral blood subsequent to chemotherapy to reach levels more suitable for use.

30. Indeed, assuming for the sake of argument, that the use of human neonatal/fetal blood as a source of stem cells for hematopoietic reconstitution had been suggested to me, the fact that normal adult peripheral blood was believed to have such a low content of long-term marrow repopulating stem cells relative to that present in bone marrow,[17] would if anything, have led me to expect that normal neonatal or fetal blood would have similarly low levels of long-term marrow repopulating stem cells and thus would not be likely to have utility for hematopoietic reconstitution. For the reasons I explained in Paragraphs 9-20 above, knowledge of cord blood cells which exhibit colony-forming abilities *in vitro*, even after replating, does not provide any information regarding

---

16 On page 244, first full paragraph, the authors state: "Leukapheresis was carried out at the time of diagnosis or two weeks after chemotherapy was discontinued."

17 See Paragraph 29 hereinabove.

the presence or quantity of long-term marrow repopulating stem cells, particularly since the ratio of stem cells (and in particular the long-term marrow repopulating stem cells) to progenitor cells in human neonatal/fetal blood is unknown.[18,19] At about the time the application leading to the '681 Patent was filed, I viewed fetal liver and adult bone marrow as being similar in that both were known to be "generating centers" for blood components, of the fetus and adult, respectively, and thus it was perhaps not unexpected in hindsight that both contained long-term marrow repopulating stem cells such that they could be used as sources of these cells for hematopoietic reconstitution. Since it was known that normal adult peripheral blood had relatively low levels of progenitor cells, and that success in using adult peripheral blood cells for hematopoietic reconstitution had generally been attained only in situations where a leukemia patient's blood expanding in response to chemotherapy was employed, this indicated to me, and I believe also to others in the art, that adult long-term marrow repopulating stem cells generally did not normally circulate in the blood, but, rather, stayed localized in the generating center, *i.e.*, bone marrow. By similar reasoning, I would not have expected that fetal or neonatal long-term marrow repopulating stem cells circulated in neonatal or fetal blood, much less in amounts sufficient to afford utility for hematopoietic reconstitution;[20] instead, I would

---

18 It was clearly unknown at about the time of the filing of the application leading to the '681 Patent. As of 1989, it was still unknown: As Linch and Brent (1989, Nature 340:676) state: "A 100-ml sample of cord blood can be expected to contain . . . progenitor cells, which should be sufficient for reconstitution after allogeneic transplantation [citation] provided that the stem-cell/progenitor-cell ratio is not appreciably less than in adult bone marrow. Only clinical studies can prove this point." To my knowledge, even as of the present date, the ratio is still unknown.

19 The ratio of long-term marrow repopulating stem cells to other cells designated "stem cells" (based on their detection by assays other than the ability to effect hematopoietic reconstitution) was and is also unknown.

20 It will thus be even more evident that I would not reasonably have expected that amounts of long-term marrow repopulating stem cells sufficient to confer utility for hematopoietic

(continued...)

have expected that such cells stayed localized in the generating center, *i.e.*, fetal liver. Indeed, had the idea of using human neonatal or fetal blood as a source of cells capable of effecting hematopoietic reconstitution in humans been disclosed to me at about the time the application leading to the '681 Patent was filed, I would have been skeptical that such idea would work. However, now that the utility of human cord blood as a source of stem cells that can be cryopreserved and used to effect hematopoietic reconstitution has been proven by many clinical successes in children having various disorders, I would not hesitate in certain situations to use cord blood stem cells for hematopoietic reconstitution, or to recommend such use of cord blood stem cells by others in the art, for example, in young children suffering from genetic disorders amenable to treatment by hematopoietic reconstitution. I believe that one of ordinary skill in the art at about November 1987 would have reasoning and conclusions substantially the same as mine set forth hereinabove in this Paragraph.

31. With respect to Ende, which is also relied upon by the Examiner, this reference does not disclose or suggest the presence of cord blood stem cells with long-term marrow repopulating ability, because there is no evidence that any long-term or complete (multilineage) repopulation of blood components was achieved. What Ende describes does not constitute an hematopoietic reconstitution, since only a brief temporary change in the patient's red blood cell phenotype was observed. The temporary change in blood antigen (M antigen) which was observed by Ende (Fig. 2, p. 3) was a red blood

---

[20](...continued)
reconstitution would be present in any single collection of human neonatal/fetal blood (*i.e.*, from a single individual); nor would one of ordinary skill in the art reasonably have expected this. Blood collected from only a single neonate or fetus would have been deemed necessary for use of human neonatal/fetal blood for hematopoietic reconstitution (except in rare circumstances such as the case of collection from identical twin neonates or fetuses), since combinations of collections from different neonates/fetuses would be avoided due to the danger of graft versus host disease and other problems stemming from histocompatibility mismatches, contamination by maternal cells, infectious agents, etc.

cell antigen. Figure 2 shows that after administration of donor M blood on March 7, 1970 (p. 2. col. 2), donor M antigen was detected on March 11, 1970 (the first time blood antigens were assayed subsequent to May 7), and donor M antigen peaked through April 14, 1970, disappearing by May 13, 1970. Thus, Ende discloses merely a temporary change in red blood cell type, as Ende himself acknowledges (see p. 1, col. 2, first full paragraph describing the "temporary allograft" achieved, and p. 3, col. 2, under "Discussion," describing "the altered red blood cell phenotype of the recipient"). Red blood cells are mature cells of the erythroid lineage, and are neither stem or progenitor cells. At about the time of the filing of the application leading to the '681 Patent, one of ordinary skill in the art would clearly view Ende as failing to provide any reasonable basis for believing that any hematopoietic reconstitution was achieved or that any therapeutic effect was achieved which would motivate cryopreservation of a composition comprising cord blood stem cells, because (1) I attribute and one of ordinary skill in the art would attribute the transient change in red blood cell antigen which was observed to the lingering presence of red blood cells originally in the transfused blood (or possibly produced from progenitor cells originally in the transfused blood); there is no information which would yield a reasonable expectation that such a change in red blood cell antigen was due to the functioning presence of a stem cell in the transfused blood; and (2) there is no information indicating that any broad (multilineage), long-term reconstitution of hematopoietic function was achieved, without which there is absolutely no indication of the presence of a long-term marrow repopulating stem cell. Short-term provision of red blood cells such as is disclosed by Ende does not motivate one to cryopreserve cord blood, since routine transfusions of noncryopreserved blood or separated red blood cells (*e.g.*, from blood banks) routinely fulfilled any need for red blood cell replacement at the

time the application leading to the '681 Patent was filed. Thus, Ende does not provide any motivation to cryopreserve a composition containing cord blood stem cells.

32. In view of Paragraphs 9-30 above, I conclude that none of the references cited by the Examiner, alone or in combination, provide any motivation to cryopreserve a composition containing human neonatal or fetal blood stem cells. As described above, with respect to Ueno et al., Prindull et al. and Nakahata et al., the art did not recognize *in vitro* assays (*e.g.*, assays detecting cells in human cord blood which have the ability to form colonies *in vitro*, before or after *in vitro* replating) as indicative of the presence of long-term marrow repopulating stem cells that confer utility for hematopoietic reconstitution. With respect to Moretti et al., Fliedner et al., Löwenberg, Gorin, Karp et al., and Korbling et al., teachings that bone marrow, fetal liver or "rebounding" adult peripheral blood can contain long-term marrow repopulating stem cells so as to confer utility for hematopoietic reconstitution does not indicate the presence of human neonatal or fetal blood stem cells conferring such utility because knowledge of the existence of long-term marrow repopulating stem cells from sources other than human neonatal or fetal blood does not yield any information regarding whether human neonatal or fetal blood contains such stem cells. With respect to Ende, knowledge that cord blood can be used in a transfusion also sheds no light on the presence, much less potential utility, of any marrow repopulating cord blood stem cells. None of the other references cited by the Examiner remedy these deficiencies in teachings.

33. In view of the foregoing, it is clear that none of the combinations of references cited by the Examiner motivate the cryopreservation of human fetal or neonatal blood, or a composition containing human neonatal or fetal blood stem cells, because for the reasons stated above, they do not lead one of ordinary skill in the art to a reasonable

expectation that human neonatal or fetal blood contains long-term marrow repopulating stem cells so as to provide utility for hematopoietic reconstitution.

34. In this and the following Paragraphs, I describe how the claimed invention fulfills needs in the art which were recognized and persistent over a long length of time, and that attempts to fulfill these needs had been repeatedly investigated by the art without satisfaction, and that, in contrast, the claimed invention of the '681 Patent provides a source of hematopoietic reconstituting stem cells with an ability to satisfy these needs that is superior to the alternatives which had been investigated by the art.

35. The need for a safe, efficacious source of stem cells capable of effecting hematopoietic reconstitution has been recognized in the art since at least the 1950's, when bone marrow began to be studied for use in hematopoietic reconstitution, in order to reconstitute the hematopoietic system of cancer patients whose hematopoietic system had been destroyed by irradiation and/or chemotherapy. From the 1950's until the time the application leading to the '681 Patent was filed, bone marrow was the source of stem cells predominantly used in hematopoietic reconstitution, since no wholly satisfactory alternatives had been found. Bone marrow reconstitution has always suffered from the recognized drawbacks that collection of bone marrow cells is an invasive procedure, posing some risk to the donor, and is expensive and laborious. Additionally, autologous bone marrow transplantation has the long-recognized disadvantages of the sick or suboptimal condition of the donor, and the threat of marrow contamination with malignant cells in cases where the patient suffers from cancer. Allogeneic (non-autologous) bone marrow transplantation has the long-recognized disadvantages of the difficulty in finding a suitable (histocompatible) donor (necessary to avoid lethal GVHD), and the problem of GVHD occurrence even when using at least partially matched donors.

Indeed, due to the heterogeneity of HLA antigens (the antigens which govern histocompatibility), it is often the case that suitable donors can't be found, or can't be found in time to avoid patient death. Even if suitable patients are registered in the National Marrow Donor Program (a registry of HLA-typed persons who volunteer to donate bone marrow to recipients who don't have a suitable sibling donor), death and illness can cause unavailability of the prospective donor when the need for his bone marrow has arisen. Locating, contacting, and counseling a donor is often time-consuming and expensive, as is the process of obtaining the donor marrow, forwarding it to the transplant center, and coordinating the hospitalization and collection. My personal experience has been that it often takes several months to find a suitable bone marrow donor for a patient with leukemia; oftentimes, that leukemia patient does not have several months to live. All of the foregoing disadvantages had been recognized in the art for over 20 years by the time the application leading to the '681 Patent had been filed.

36. Since at least the 1970's and into the 1980's, fetal thymus, fetal liver, and adult peripheral blood had been investigated as alternatives to bone marrow, as sources of stem cells with utility for human hematopoietic reconstitution, in the hope that they would overcome the recognized drawbacks associated with the use of bone marrow. However, the art did not consider any of these other sources to be satisfactory alternatives to the use of bone marrow at the time the application leading to the '681 Patent was filed.

37. Fetal thymus and fetal liver as sources of stem cells for hematopoietic reconstitution were found to suffer from the recognized drawbacks of high failure rates due to lack of long-term or complete hematopoietic repopulation and/or GVHD, as well as the evident problem of limited availability and accessibility. Fetal thymus and liver had to be surgically removed from aborted fetuses. Aborted fetuses could not be counted

on to be available. Even if available, one could not count on obtaining a liver or thymus from a fetus old enough to have a liver or thymus large enough to afford a sufficient number of long-term marrow repopulating stem cells. These problems had led to the substantial rejection of the use of fetal thymus and/or liver for hematopoietic reconstitution by the time the application leading to the '681 Patent was filed.

38. Normal adult peripheral blood, due to its low levels of circulating stem cells, does not have any practical utility for effecting hematopoietic reconstitution, and thus was not deemed a practical alternative to bone marrow. Some success was achieved in the prior art in the use of adult peripheral blood mononuclear cells of certain leukemia patients; however, this success was largely viewed as due to the fact that the blood was collected while "rebounding" after chemotherapy (*i.e.*, while the stem and progenitor cell levels were believed to be increased in response to chemotherapy).[21]

This procedure was thus recognized to suffer from the drawbacks of potential malignant contamination of the peripheral blood cells collected from the patient, as well the dependency of collection upon patient availability and condition (*i.e.*, involving collection from a patient with a sick or suboptimal condition) and limited as to when the cells could be collected at a time when the desirability of hematopoietic reconstitution for the particular patient was already recognized. It also involved intensive leucopheresis for collection of sufficient cells, which is time-consuming and expensive. Furthermore, the procedure suffered from variability in amount of long-term marrow repopulating stem cells in the collected blood, indicated by problems of incomplete or unstable hematopoietic repopulation presumably due to low levels of such cells.

---

[21] See Paragraphs 26-29 hereinabove.

39. In contrast, the use of cryopreserved human neonatal/fetal blood stem cells overcomes these drawbacks long recognized in the art. Cryopreserved human neonatal/fetal blood stem cells fulfill the long-recognized needs in the art for a source of stem cells (1) capable of safely and effectively carrying out human hematopoietic reconstitution, without severe GVHD; (2) with reduced potential for contamination by a patient's malignant cells or affliction with other disorders or infections associated with the patient or with adult tissue; (3) easily obtainable, without entailing an invasive surgical procedure with its attendant costs and risks; (4) not dependent on having a patient healthy enough to undergo the procurement procedure; (5) inexpensive; and (6) abundant/widely available. By way of example, placental and cord blood are available at every birth and were routinely discarded in the prior art. This great availability makes feasible the establishment of banks of cryopreserved neonatal/fetal blood stem cells, containing suitable donors for all population groups, even those minority populations presently underrepresented in the bone marrow registry, so as to expedite greatly finding and obtaining suitable donors of cells for hematopoietic reconstitution. Furthermore, frozen neonatal/fetal blood (or stem cell-containing fractions thereof) can be easily shipped and thawed for use, reducing the coordination, time, delay and expense associated with obtaining collected bone marrow. In addition, human neonatal/fetal blood has not been subjected to any detrimental effects associated with the aging process, for example, it should generally have a lower risk of containing infectious agents or disease than adult sources of long-term marrow repopulating stem cells. With respect to autologous use, the blood is collected at a time that is generally well prior to the onset of the illness (*e.g.*, cancer) desired to be treated by hematopoietic reconstitution, and can be stored for later use. In its simplest aspect, the human neonatal/fetal blood can be easily obtained by

direct drainage from the umbilical cord, without the need for any invasive, surgical procedure or anesthesia. Numerous publications in the art have now appeared, documenting the clinical successes achieved using human cord blood in children suffering from various disorders, and demonstrating that cryopreserved human neonatal/fetal blood stem cells can serve as a source of long-term marrow repopulating stem cells that safely and effectively carry out hematopoietic reconstitution, without problematic GVHD.

40. I hereby declare further that all statements made herein of my own knowledge are true and that all statements on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the captioned patent.

Date: 9/14/94

Irwin D. Bernstein, M.D.

Attachments:

Exhibit A: Rowley et al., 1985, Exp. Hematol. 13:295-298

Exhibit B: Gordon et al., 1985, Leukemia Research 9:1017-1021

Exhibit C: Siena et al., 1985, Blood 65(3):655-662

Exhibit D: Yeager et al., 1986, N. Engl. J. Med. 315(3):141-147

Exhibit E: Kaizer et al., 1985, Blood 65(6):1504-1510

Exhibit F: Andrews et al., 1986, Blood 68(5):1030-1035

Exhibit G: Jacobsen et al., 1979, Cell Tissue Kinet. 12:213-226

Exhibit H: Richman et al., 1976, Blood 47:1031-1039